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Knockdown of TCTN1 Strongly Decreases Growth of Human Colon Cancer Cells

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Background: Tectonic family member 1 (TCTN1), a member of the tectonic family, is involved in several developmental processes and is aberrantly expressed in multiple solid tumors. However, the expression and regulation of TCTN1 in human colorectal cancer (CRC) is still not clear.

Material/Methods: The expression of TCTN1 mRNA was first explored by using Oncomine microarray datasets. TCTN1 expression was silenced in human CRC cell lines HCT116 and SW1116 via RNA interference (RNAi). Furthermore, we investigated the effect of TCTN1 depletion on CRC cell growth by MTT, colony formation, and flow cytometry *in vitro*.

Results: In this study, meta-analysis showed that the expressions of TCTN1 mRNA in CRC specimens were significantly higher than that in normal specimens. Knockdown of TCTN1 expression potently inhibited the abilities of cell proliferation and colony formation as determined. Flow cytometry analysis showed that depletion of TCTN1 could cause cell cycle arrest at the G2/M phase. In addition, Annexin V/7-AAD double-staining indicated that TCTN1 silencing promoted cell apoptosis through down-regulation of caspase 3 and Bcl-2 and upregulation of cleaved caspase 3 and PARP.

Conclusions: Our results indicate that TCTN1 may be crucial for CRC cell growth, providing a novel alternative to target therapies of CRC. Further research on this topic is warranted.

MeSH Keywords: Apoptosis • Cell Proliferation • Colonic Neoplasms • Genes, Neoplasm • RNA, Small Interfering

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Background

Although the death rate of colorectal cancer (CRC) patients has been decreasing in the last several years, CRC is still one of the most common cancers, with a high morbidity all around the world [1]. In the USA, 136 830 people were diagnosed with CRC and 50 310 died from it in 2014, which means CRC is the third most common cancer and is the leading cause of cancer deaths in the USA [2]. In China, the CRC situation is not as bad as in the USA, but has recently been worsening [1]. Even after surgical removal, the high 2-year recurrence rate (around 75%), and the low 5-year survival rate (only about 26.8%) are still unsolved serious clinical problems [3]. Thus, developing new therapies for the treatment of advanced colon cancer based on the mechanism of colorectal carcinogenesis remains important.

Colorectal carcinogenesis is a complex process involving various molecular pathways with associated and diverse changed expressions of oncogenes and anti-oncogenes. Currently, more and more oncogenes and anti-oncogenes have been identified in studies of CRC, such as laminin β-1 (LAMB1) and GRIM-19, which have been shown to be promising novel biomarkers in CRC and are closely correlated with the disease course of CRC [3,4]. Although there have already been many such findings reported, the confirmed related genes are only a small part of all the molecular targets resulting in CRC. Thus, disordered genes of CRC remain to be discovered through the investigation of its underlying molecular mechanisms to develop more effective therapies for CRC.

Tectonic family member 1 (TCTN1), a novel regulator of the Hedgehog pathway, participates in a variety of developmental processes [6] and encodes a family of secreted and transmembrane evolutionarily conserved proteins that play a crucial role in various biological processes, including cellular differentiation [8], angiogenesis [9], apoptosis [10], and modulation of the immune response [11]. In addition, a recent report has shown that TCTN1 enters into the ciliopathy-related protein complex and interacts with some other proteins linked to ciliopathies [12]. Moreover, researches have reported that TCTN1 is involved in the growth of malignant cells in prostate cancer [17] and gastric cancer [19], as well as in glioblastoma cells [18]. However, the function of TCTN1 in human CRC has never been reported.

In this study, we investigated the effect of TCTN1 on CRC growth and progression. We first investigated the expression level of TCTN1 in CRC tissue by Oncomine data mining to identify the molecular target of TCTN1 in CRC cell lines. Subsequently, we described the influence of TCTN1 silencing on cell proliferation, cell cycle, and apoptosis in human CRC cells. The results show that TCTN1 might play a significant role in CRC tumorigenesis and could be a practical target for effective treatments of CRC in the future.

Material and Methods

Cell culture

CRC cell lines HCT116 and SW1116 and human embryonic kidney cell line 293T (HEK293T) were obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). Among these 3 cell lines, HCT116 cells were cultured in RPMI-1640 (Hyclone SH30809.01B+) supplemented with 10% FBS (fetal bovine serum, Biowest, S1810). SW1116 and 293T cells were cultured in DMEM (Hyclone, SH30243.01) supplemented with 10% FBS (Biowest, S1810). All cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Knockdown of TCTN1 expression with shRNA

According to the sequence of TCTN1 (NM_001082537.2), we designed the sequence of shRNA: 5'--GCTCAGATGCATCAGT TCCTTCTCGAGAAGGAACTGATGCATCTGAGCTTTTTT-3'. The control shRNA sequence was 5'-TTCTCCGAACGTGTCACGT-3'. Both of the shRNAs were cloned into the pFH-L vector containing a green fluorescent protein (GFP) gene reporter (Shanghai Hollybio, China) between the restriction enzyme cutting sites *NheI* and *PacI*. The recombinant pFH-L vector and the helper plasmids (pVSVG-I and pCMV ΔR8.92, Shanghai Hollybio, China) were co-transfected with the 293T cell line to generate lentivirus using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. The constructed lentiviruses were named shCon and shTCTN1. Then, the lentiviruses were filtered and viral titers were determined. Multiplicities of infection (MOI) of 30 and 25 were achieved in HCT116 and SW1116 cells, respectively. The cells (50000 cells per well) were infected for 72 h by different lentiviruses (shCon and shTCTN1) and the infection efficiency was measured by observing the number of GFP-positive cells under a fluorescence microscope. The expression of TCTN1 silencing was confirmed by quantitative PCR and Western blotting.

Quantitative real-time PCR (qPCR)

The qPCR was performed in HCT116 and SW1116 cells after infection for 5 days, using BioRad Connet RT-PCR platform (CFX96, BioRad, California, USA). The qPCR primers were TCTN1: (forward) 5'-CCTTTGCGTGAATGTTGTTC-3' and (reverse) 5'-AGAGGGACTGGCTGGGTATT-3', and β-actin: (forward) 5'-GTGGACATCCGCAAAGAC-3' and (reverse) 5'-AAAGGGTGTAACGCAACTA-3'. The qPCR reaction system contained 2×SYBR premix ex taq 10 µl, cDNA 5 µl, forward and reverse primers (2.5 µM) 0.8 µl and ddH_2O 4.2 µl. The qPCR

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procedure was conducted as follows: step 1: initial denaturation at 95°C for 1 min; step 2: denaturation at 95°C for 5 s; step 3: annealing extension at 60°C for 20 s, with 40 cycles total. The expression of detected genes was analyzed by the $2^{-\Delta\Delta Ct}$ method.

Western blotting

We lysed the cells after lentivirus infection for 7 days to get the total protein. The lysis buffer of total protein contained 2×SDS Sample Buffer: 100 mM Tris-Hcl (pH 6.8), 4% SDS (SB0485-500g, Sangon, Shanghai, China), 10 mM EDTA (Sangon, Shanghai, China), and 10% Glycine. The protein solution (30 μg) was separated by SDS-PAGE and transferred to PVDF membranes. After blocking, the membranes were probed with primary antibody: rabbit anti-TCTN1 antibody (dilution 1:500, SAB3500518, Sigma, USA), rabbit anti-caspase 3 antibody (dilution 1:500, 19677-1-AP, Proteintech, USA), rabbit anti-cleaved caspase 3 antibody (dilution 1:500, 9661, Cell signaling, USA), rabbit anti-PARP antibody (dilution 1:500, 9542, Cell signaling, USA), and rabbit anti-Bcl-2 antibody (dilution 1:1000, 2876, Cell signaling, USA) at 4°C overnight. The positive control protein was rabbit anti-GAPDH antibody (dilution 1: 100 000, 10494-1AP, Proteintech, USA). Then, we probed the membrane with secondary antibody: goat anti-rabbit horseradish peroxidase (HRP) antibody (dilution 1:5000, SC-2054, Santa Cruz, USA) at room temperature for 2 h. After incubation, the membrane visualized by use of the ECL Western Blotting Substrate kit (Pierce, USA).

Cell viability

The MTT assay was used to examine the effects of TCTN1 silencing on cell proliferation in HCT116 and SW1116 cells. After 72 h of lentivirus infection, the cells were cultured in 96-well plates with the concentration of 3000 cells each well. Subsequently, we added 20 μl of 5 mg/ml MTT (M2128, Sigma, USA) per well and cultured them for 4 h. Then, 100 μl acidified isopropanol containing 10% SDS, 5% isopropanol, and 0.01 mol/L HCL was added and cells were further cultured overnight at 37°C to stop the reaction. The OD number was determined at 595 nm with a microplate reader.

Cell colony formation

After lentivirus infection for 72 h, the HCT116 cells were seeded into a 6-well plates with 600 cells per well. The cells were cultured for 8 days at 37°C, washed with PBS twice, and fixed with 4% paraformaldehyde for 10 min at room temperature. After being washed with PBS twice, the fixed cells were stained by crystal violet for 5 min, washed with PBS, and air-dried. Then, the cell colonies were observed and counted under a microscope.

Cell cycle analysis

Flow cytometry was performed to investigate the influence of TCTN1 silencing on the cell cycle distribution. After lentivirus infection for 5 days, HCT116 cells were seeded into 6-cm dishes at a density of 80 000 cells per dish and cultured for 48 h. Then, the cells were harvested, washed by pre-cooling PBS, and fixed by 75% ethanol (10009269, Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) overnight at 4°C. HCT116 cells were rewashed and stained in 500 μl PI buffer (C1052, Beyotime Biotechnology, Shanghai, China) for 1 h at 37°C in the dark. Finally, flow cytometry was performed to detect the cell cycles.

Cell apoptotic assay

The Annexin V-APC/7-AAD double-labeling kit (KeyGEN, Nanjing, China) was used to detect cell apoptosis after TCTN1 silencing. Briefly, HCT116 cells were seeded into 6-cm dishes at a density of 80 000 cells per dish after 5 days of lentivirus infection and continued to culture for 48 h. Then, the cells were collected, washed, and stained by Annexin V-APC/7-AAD according to the manufacturer's instructions. Finally, cells were analyzed by use of a FACS-Calibur (BD Biosciences, USA). The proportions of cells were divided into 4 groups as viable cells ($APC^-/7\text{-}AAD^-$), necrotic cells ($APC^-/7\text{-}AAD^+$), early apoptosis ($APC^+/7\text{-}AAD^-$), and late apoptosis ($APC^+/7\text{-}AAD^+$).

Oncomine database analysis

A meta-analysis on the online Oncomine Expression Array database (www.oncomine.org) was conducted to compare the differential expression of TCTN1 between CRC and normal tissues. The search terms were used: "TCTN1", "Colorectal Cancer", "mRNA" and "Cancer *vs.* Normal Analysis". A total of 4 datasets were extracted according to the results of screening, including Sabates-Bellver Colon (GEO accession GSE8671) [20], Gaedcke Colorectal (GEO accession GSE20842) [21], Hong Colorectal (GEO accession GSE9348) [22], and TCGA Colorectal (The Cancer Genome Atlas-Colon and Rectum Adenocarcinoma Gene Expression Data, *http://tcga-data.nci.nih.gov/tcga/*). Each dataset was plotted using GraphPad Prism 5 software.

Statistical analysis

All the experiments were repeated 3 independent times. The differences between shTCTN1 and shCon groups were evaluated by the *t* test and expressed as the mean ±SD. The results were analyzed using SPSS17.0 software. The p-value <0.05 was considered as significance.

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Results

TCTN1 mRNA expression is up-regulated in CRC

To confirm whether the expression of TCTN1 was different between CRC and normal samples, first we queried the public Oncomine cancer database to perform the meta-analysis of TCTN1 gene expression. As shown in Figure 1A, meta-analysis of the 4 datasets revealed that TCTN1 mRNA expression was significantly higher than in the normal tissues in CRC, with a median rank of 2147.5 and P-value of 3.93E-8. Specifically, TCTN1 mRNA expression in colon adenoma (n=25, p=3.53E-5) and rectal adenoma (n=7, p=1.27E-6) showed a significant increase as compared with that in the normal tissues (n=32) in the Sabates-Bellver Colon dataset (Figure 1B). Similarly, expression of TCTN1 was remarkably elevated in rectal adenocarcinoma (n=65, p=1.52E-12) compared with the corresponding normal tissues (n=65) by using the Gaedcke Colorectal dataset (Figure 1C), and was observably higher in colorectal carcinoma (n=70, p=3.39E-8) than in normal tissues (n=12, Figure 1D) in the Hong Colorectal dataset. Furthermore, TCTN1 was significantly up-regulated in different types of CRC (cecum adenocarcinoma: n=22, p=7.85E-8; colon adenocarcinoma: n=101, p=1.80E-14; colon mucinous adenocarcinoma: n=22, p=3.01E-6; rectal adenocarcinoma: n=60, p=6.57E-14; rectal mucinous adenocarcinoma: n=6, p=0.001; rectosigmoid adenocarcinoma: n=3, p=0.025) compared with normal tissues (n=22) in the TCGA Colorectal dataset (Figure 1E). These data indicate that TCTN1 expression is disordered in CRC and might contribute to the occurrence and development of CRC.

Depletion of TCTN1 expression was successful in human CRC cell lines via lentivirus-mediated shRNA system

Because we hypothesized that TCTN1 might be involved in human CRC progression, HCT116 and SW1116 cells were then cultured and infected with different groups (Con, shCon, and shTCTN1). At 72 h post-infection, the infection efficiency exceeded 80% as measured by observing GFP-positive cells in both HCT116 and SW1116 cells, indicating a successful infection (Figure 2A).

Therefore, we further determined the efficiency of TCTN1 silencing in protein and mRNA levels through qRT-PCR and Western blotting assays in CRC cells. We found that TCTN1 mRNA expression was significantly decreased by 81.75% in HCT116 cells and 93.2% in SW1116 cells after transfection with shTCTN1 group (Figure 2B, $p<0.001$). In addition, TCTN1 protein expression in the shTCTN1 group was obviously lower than that in the shCon group (Figure 2C). Results demonstrated that the lentivirus-mediated shRNA system successfully inhibited TCTN1 gene expression.

Depletion of TCTN1 expression inhibited proliferation and colony formation capabilities in human CRC cells

To determine the role of TCTN1 in CRC cells, we investigated whether depletion of TCTN1 gene expression affected the cell growth ability. MTT assays were performed to detect cell proliferation in HCT116 and SW1116 cells after TCTN1 silencing. As shown in Figure 3A, the growth rates of HCT116 and SW1116 cells were remarkably lower in the shTCTN1 group. The number of viable cells on the 5th day was much lower in the shTCTN1 group than in the shCon group in HCT116 (8.17±0.67 *vs.* 11.40±0.60) and SW1116 (4.29±0.16 *vs.* 7.25±0.51) cells, respectively ($p<0.001$).

Furthermore, colony formation assay was carried out to confirm the effect of TCTN1 on malignant proliferation of CRC cells. We found the colony size and number were significantly lower after TCTN1 silencing (Figure 3B). The number of colonies in HCT116 cells was reduced dramatically in the shTCTN1 group as compared with that in the shCon group (2.33 ±1.53 *vs.* 83.33±2.52, $p<0.001$) (Figure 3C). These findings indicate that TCTN1 might play an important role in the growth of human CRC cells.

Depletion of TCTN1 led to CRC cell cycle arrest

We next investigated the mechanisms underlying the inhibitory effect of TCTN1 silencing on CRC cell growth. We analyzed cell cycle distribution by flow cytometry to determine cell populations at different phases (Figure 4A). As shown in Figure 4B, knockdown of TCTN1 in HCT116 cells significantly decreased the percentage of cells in G0/G1 phase (64.76±0.61% *vs.* 74.80±0.64%, $p<0.001$) and increased those in G2/M phase (22.65±0.30% *vs.* 13.63±0.92%, $p<0.01$) in comparison with the shCon group. More importantly, the sub-G1 population (representing apoptotic cells) was significantly increased in the shTCTN1 group (5.97±0.44% *vs.* 0.29±0.12%, $p<0.01$) as compared with the shCon group (Figure 4C). These findings suggest that down-regulation of TCTN1 affects CRC cell cycle progression.

Depletion of TCTN1 promoted CRC cell apoptosis

To analyze whether TCTN1 silencing regulates apoptosis in CRC cells, Annexin V-APC/7-AAD double-staining was carried out and the percentage of cells in the 4 stages (viable cells, necrotic cells, early apoptosis, and late apoptosis) was determined by flow cytometry (Figure 5A). As shown in Figure 5B, the proportion of apoptotic cells in the shTCTN1 group was significantly higher than that in the shCon group and Con group in HCT116 cells. These results suggest that knockdown of TCTN1 expression induces apoptosis in CRC cells, which is conducive to inhibition of cell growth.

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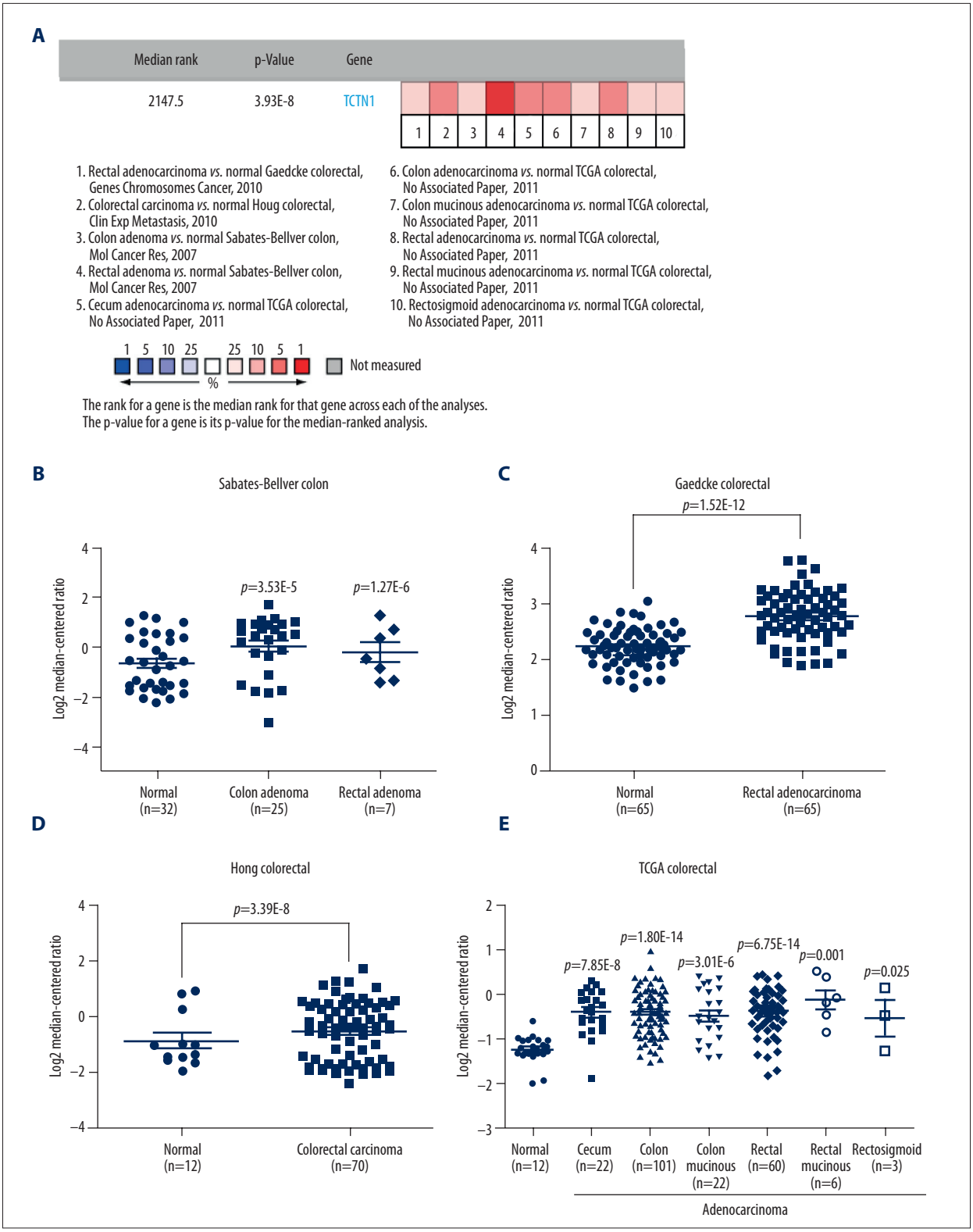

Figure 1. Bioinformatics analysis of TCTN1 in CRC cancer. (**A**) Four microarray datasets on TCTN1 mRNA expression between CRC and normal tissues were included in the meta-analysis via Oncomine cancer microarray database. (**B–D**) The specific expression level of TCTN1 gene were obtained from Sabates-Bellver Colon dataset, Gaedcke Colorectal dataset, Hong Colorectal dataset, and TCGA Colorectal dataset shown as scatter diagram, respectively.

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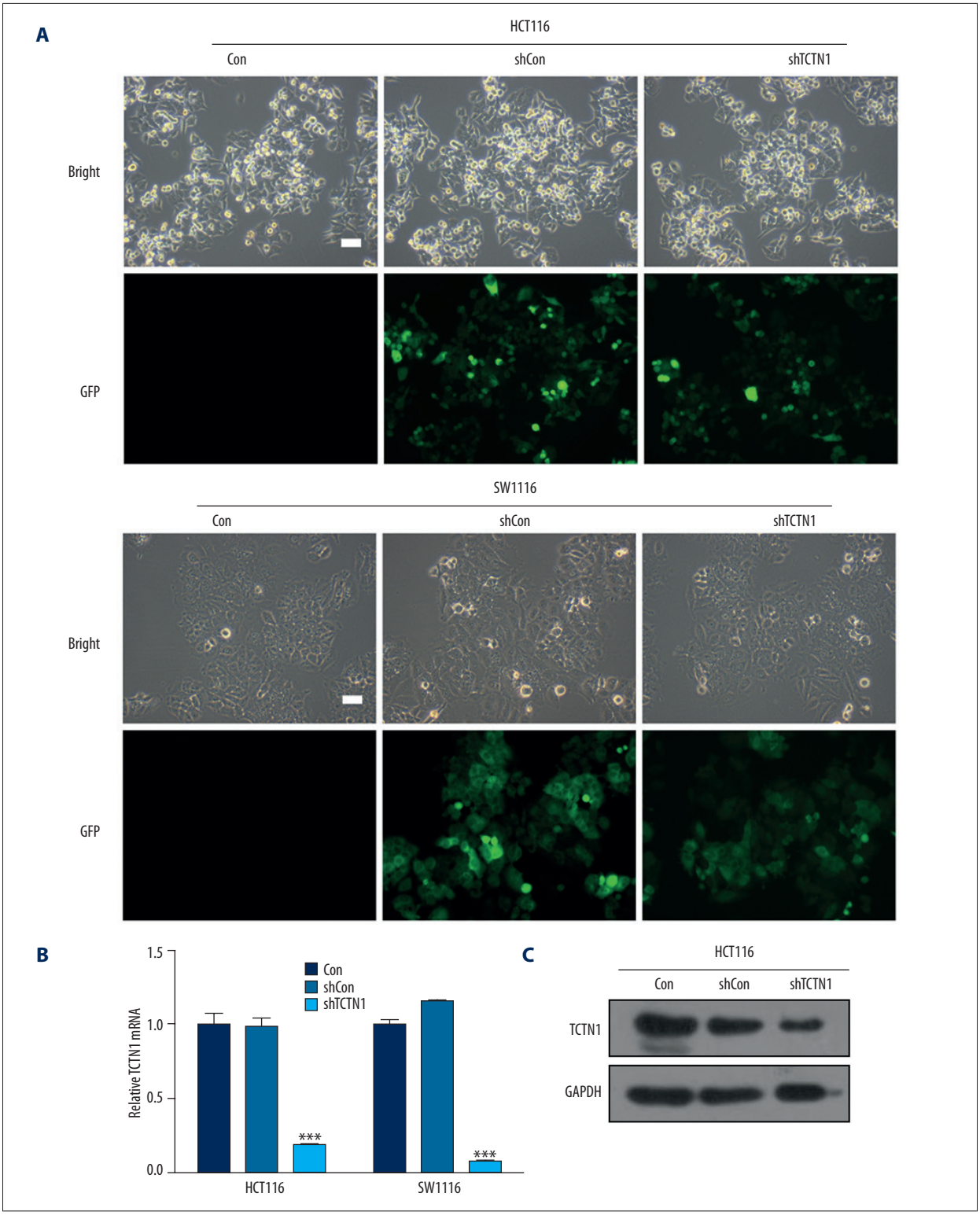

Figure 2. Depletion of TCTN1 expression was successful in human CRC cell lines via lentivirus-mediated shRNA system. (**A**) GFP expression was observed to assess the infection efficiency in shCon and shTCTN1 groups in HCT116 and SW1116 cells. (**B, C**) The expression of TCTN1 in mRNA and protein levels was detected by quantitative real-time PCR (qRT-PCR) and Western blotting assays in TCTN1-knockdown group in CRC cells, respectively. Scale bar, 100 μm; *** p<0.001, compared with shCon group.

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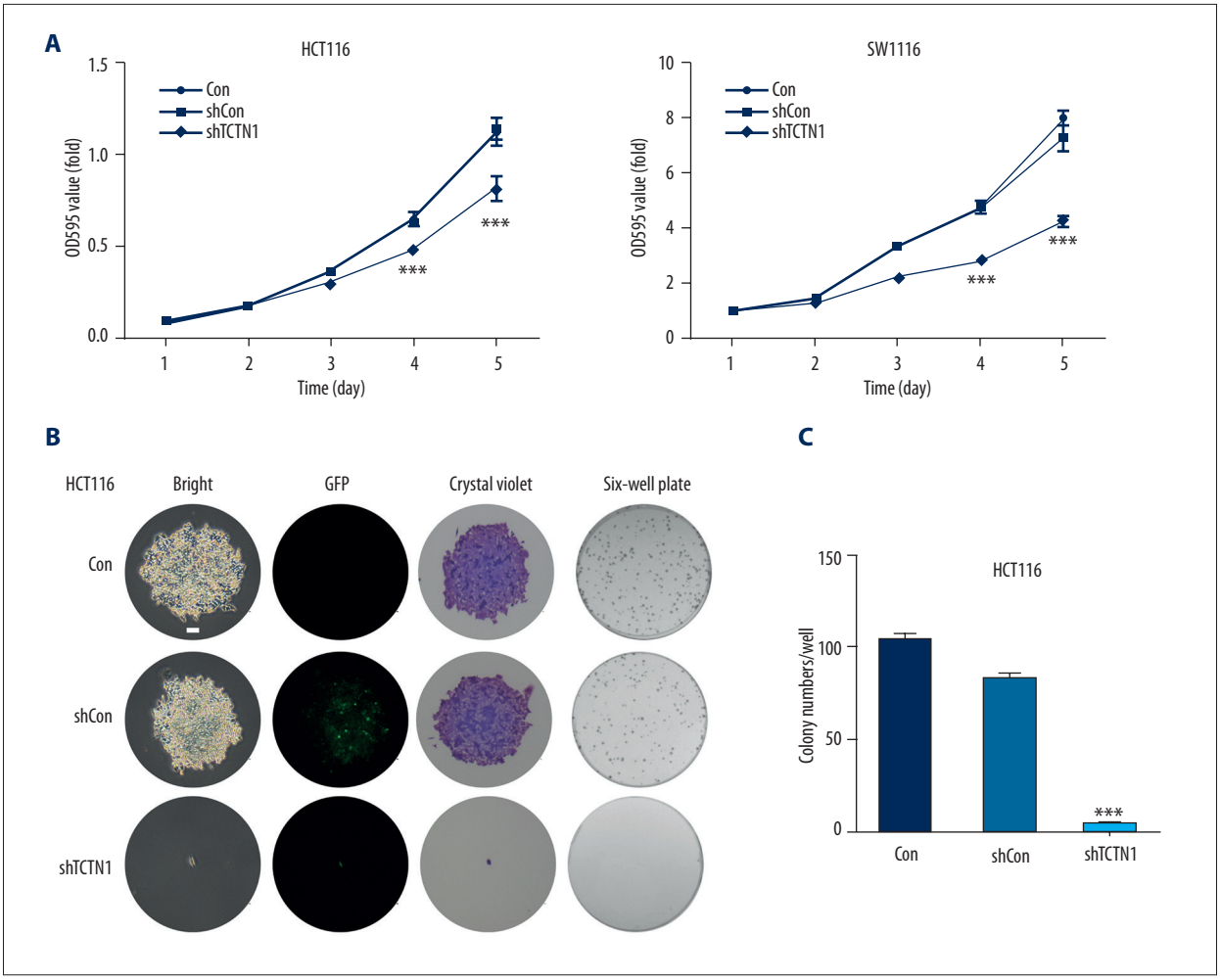

Figure 3. Depletion of TCTN1 expression inhibited human CRC cell growth. (**A**) The growth rates were measured after down-regulation of TCTN1 in HCT116 and SW1116 cells by MTT assay. (**B**) The abilities of colony formation in the size and number are shown in HCT116 cells after infection with shTCTN1. (**C**) Statistical analysis of the colony numbers in Con, shCon, and shTCTN1 groups. Scale bar, 25 μm; *** p<0.001, compared with shCon group.

Decline of TCTN1 affected the expression of cell apoptosis-related proteins in HCT116 cells

To further investigate the potential mechanism by which decreased TCTN1 inhibits cell growth, the expression patterns of cell apoptotic molecules Bcl-2, caspase-3, and PARP were analyzed by Western blot assay. As shown in Figure 5C, we found that the expression levels of pro-caspase-3 and Bcl-2 were obviously decreased in the HCT116 cells lacking TCTN1. Correspondingly, the protein levels of cleaved caspase-3 and PARP were significantly increased in the shTCTN1 group. The above results suggest that knockdown of TCTN1 affects CRC cell growth via the regulation of Bcl-2, caspase-3, and PARP expression.

Discussion

Colorectal cancer (CRC) is one of the most universal malignancies, and its pathogenic mechanism remains unclear. In recent years, the treatment of CRC has transformed surgical excision alone into multidisciplinary combination therapy, wherein drug combination therapy and prognosis evaluation are important strategies for treating CRC [23,24]. Moreover, molecular targeting therapy is also a promising method for treating CRC. Thus, more potential targeted biomarkers for CRC are urgently needed. The present study focused on TCTN1 as a novel underlying targeting molecule for CRC, which is up-regulated in CRC specimens and can regulate the growth of CRC cells.

TCTN1 was identified as a novel oncogene involved in developmental processes, Hedgehog pathway, and the role of primary cilium [6,12]. In the developmental processes, the regulators

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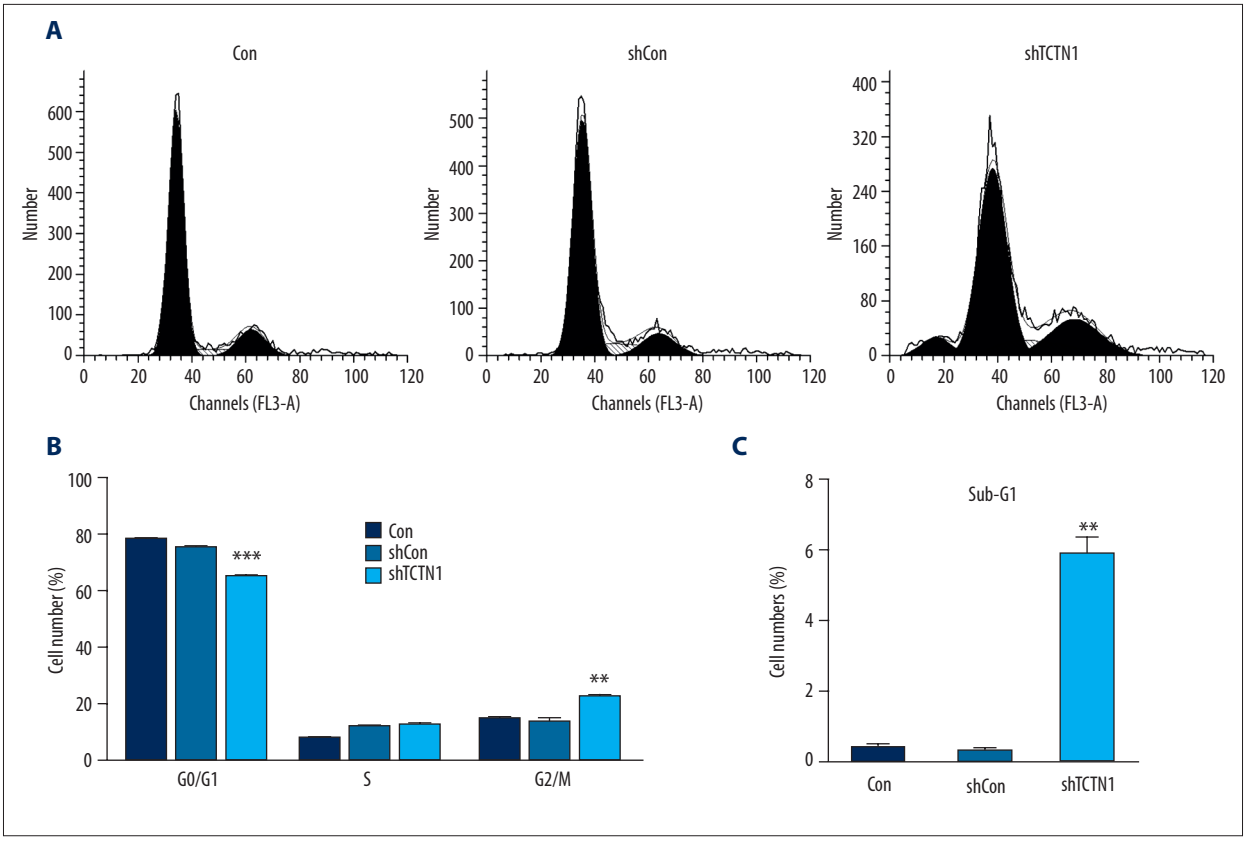

Figure 4. Depletion of TCTN1 arrested cell cycle progression. (**A**) Cell cycle progression was assayed by flow cytometry in 3 different groups: Con, shCon, and shTCTN1. (**B**) Statistical analysis of the percentage of CRC cells in 3 different phase (G0/G1, S, and G2/M phases). (**C**) Statistical analysis of the percent of cells in sub-G1 phase. ** p<0.01; *** p<0.001, compared with shCon group.

are often destroyed in oncogenesis [25]. Likewise, Hedgehog pathway and the primary cilium also participate in tumorigenesis [15,26]. Thus, we hypothesized that TCTN1 also may be correlated with tumor formation. Currently, there has been no report about the role of TCTN1 in CRC. Hence, our study aimed to reveal the possible role of TCTN1 in CRC progression. The Oncomine cancer microarray database allows investigation of gene expression between cancer specimens and normal specimens [27,28]. Therefore, we explored the expression trend of TCTN1 between different CRC subtypes and normal tissues in 4 different datasets. We found that TCTN1 mRNA expression in CRC tissues was dramatically higher than that in the normal tissues, which was in concordance with previous finding that TCTN1 is significantly overexpressed in human glioblastoma [18], suggesting that TCTN1 may play an important role during carcinogenesis.

Oncomine clinical database analysis suggested TCTN1 may play an important role during the carcinogenesis of CRC, but there has been no related report on its biological role in CRC. Therefore, we carried out experiments *in vitro* in 2 CRC cell lines HCT116 and SW1116 by use of knockdown of TCTN1. Consequently, we observed that depletion of TCTN1 clearly hampered the abilities of cell proliferation and colony formation. Moreover, TCTN1 silencing arrested cell cycle at G2/M phase and promoted cell apoptosis. Consistent with our results, it has also been observed that TCTN1 knockdown inhibits cell growth and induces G2/M phase arrest in different types of malignant tumor cells [17,19,29,30], suggesting that TCTN1 may in part regulate cell growth in CRC.

Further apoptosis analysis via Western blotting assay showed apoptotic cells were accumulated after TCTN1 silencing and the cleavage of caspase-3 and PARP protein expression were increased. Furthermore, knockdown of TCTN1 obviously reduced the expression of Bcl-2, which is a classic member of the Bcl-2 family of anti-apoptotic proteins [31]. Apoptosis is a process of programmed cell death and plays an important role in the growth of cancer cells [32]. Caspase-3, as an effector caspase, can regulate the caspase cascade, which is a major element of cell apoptosis [33]. Furthermore, PARP is one of the most commonly used tools to detect apoptosis because PARP is a particular substrate that can be proteolyzed by the activation of caspase 3, further promoting cell apoptosis [34–36].

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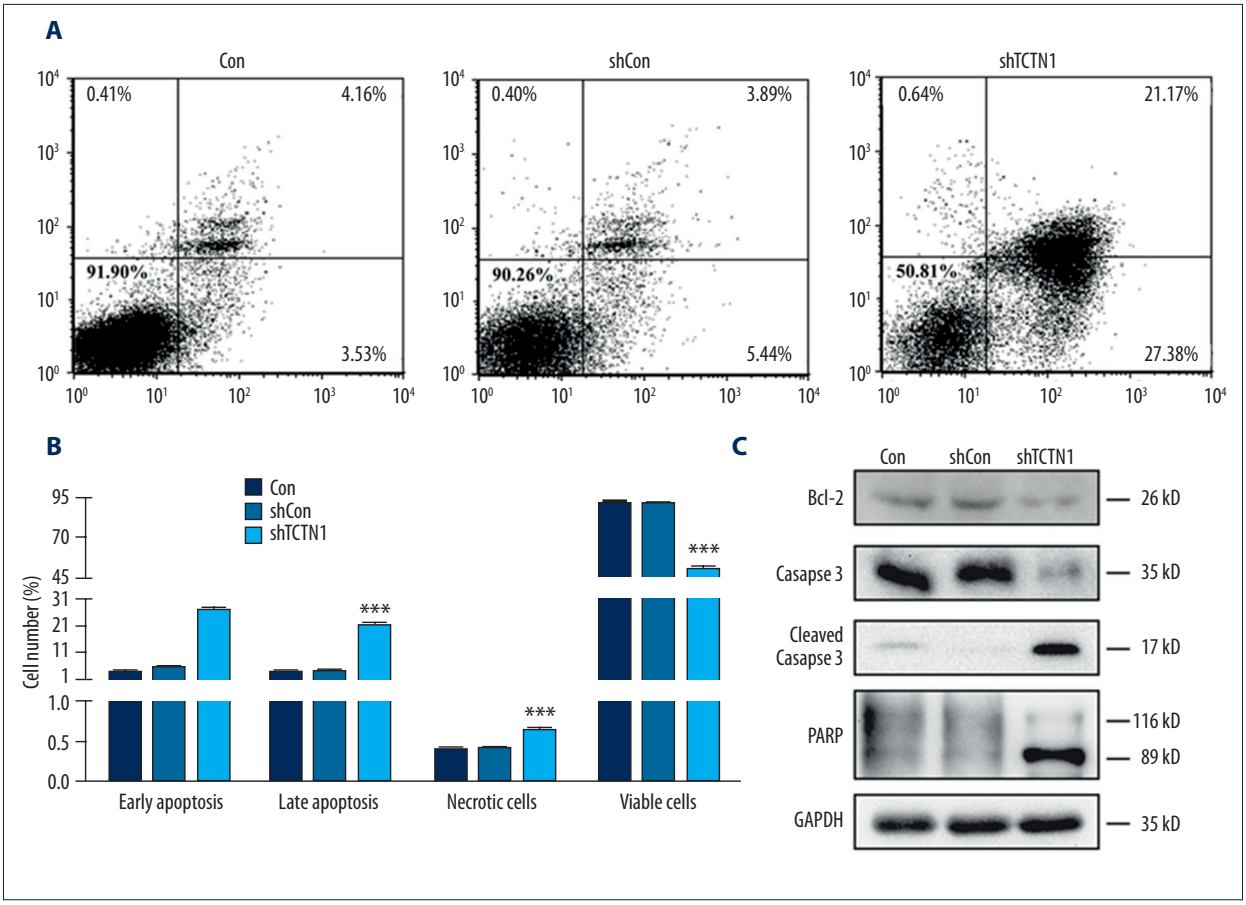

Figure 5. Depletion of TCTN1 induced CRC cell apoptosis. (**A**) Cell apoptosis was analyzed by flow cytometry in 3 different groups: Con, shCon, and shTCTN1. (**B**) Statistical analysis of the percentage of cells in 4 different stages (viable cells, necrotic cells, early apoptosis, and late apoptosis). (**C**) Apoptosis-related molecules were detected by Western blot after TCTN1 knockdown in of HCT116 cells. *** p<0.001.

Conclusions

We preliminary described that knockdown of TCTN1 suppressed cell growth by inducing cell cycle arrest and apoptosis in CRC. Our findings to some extent indicate that TCTN1 might act as an underlying oncogene that plays a crucial role in the occurrence and development of CRC.

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